REMARKS

Interview

Applicants would like to thank Examiner Bertoglio for the phone conference with Applicants's representative on March 17, 2009. During the interview, the bases for the rejections were discussed with regard to the indefiniteness of the claims and the applicability of the cited art. In view of that conversation, Applicants have amended the claims and present the following remarks.

Status of the Claims

Claims 13-24 are currently pending and under examination. Claims 1-12 and 25 have been canceled without prejudice or disclaimer to the subject matter claimed therein.

Amendment to the Claims

Claim 13 has been amended. Representative support can be found in the specification at page 10, line 7. The amendments to the claims do not add prohibited new matter.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 13-24 are rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to distinctly point out and claim the subject matter regarded as the invention.

The Office Action alleges that claim 13 is vague and indefinite. Without acquiescing to the propriety of the rejection and solely to advance prosecution of the application, claim 13 has been amended. Claim 13, as it stands, is not vague and indefinite. Applicants respectfully point out that the treated nucleus can be transferred into the oocyte when the oocyte is in interphase or metphase, but activation requires that the oocyte be in metaphase. It is therefore respectfully requested that this rejection be withdrawn.

Rejection under 35 U.S.C. § 103(a)

Claims 13-24 are rejected under 35 U.S.C. § 103(a), as allegedly being obvious under Wangh (U.S. Patent 6,753,457) in view of Samocha-Bone (Molecular Human Reproduction 4: 133-137, 1998).

The Office Action alleges it would be obvious to combine the methods of reprogramming nuclei in somatic cells as disclosed by Wangh with the methods for treating sperm nuclei disclosed in Samocha-Bone to arrive at the claimed invention.

Wangh discloses methods that activate non-dividing diploid cells with the incubation of isolated diploid or haploid nuclei with an activating egg extract, a feature that is not present in the claimed invention. The claims use closed language in reciting the steps of the claimed method of preparing a reconstituted embryo. Accordingly, the step of using activated egg extract is excluded.

Samocha-Bone discloses methods for nuclear decondensation of spermatozoa with the use of heparin and reducing agents. The Office Action relies on Samocha-Bone to disclose the use of polyanions for causing sperm nuclear swelling. Samocha-Bone, like Wangh, discloses nuclear swelling with the presence of a reducing agent. The claimed invention however, is a complete and closed series of steps useful for preparing a reconstituted embryo. Accordingly, the use a reducing agent is excluded.

With regard to the combination of the references, Wangh discloses methods for diploid nuclei whereas the present invention is directed to somatic haploid nuclei. Diploid and haploid nuclei, and in particular sperm nuclei are very different. Enclosed with this response is a paper which describes the properties of sperm nuclei (Hennig, DNA Packaging in Sperm, Encylopedia of Life Sciences, 2005). This paper, in the first sentence of the introduction states:

> "In mature male germ cells [sperm], the chromatin conformation differs from that of other cell types. The deoxyribonucleic acid (DNA) is packed more tightly than and in a way different from metaphase chromosomes[of all other cell types]. This is achieved with the aid of male germline-specific chromosomal proteins"

Therefore, it was known and established that sperm nuclei contain 50% less DNA than a diploid nucleus. Sperm nuclei are packaged and arranged in an entirely different conformation from that of haploid cells. The DNA in a sperm nuclei is more tightly packaged than normal diploid metaphase chromosomes, which consequently causes the haploid nucleus to be metabolically

inactive. It was further understood in the art that these differences are the result of male germline-specific proteins and accordingly cannot occur in non-germline and non-male cells. Therefore, a haploid nuclei from a spermatozoa and a diploid nuclei isolated from a somatic donor cell are not the same and cannot be treated in a similar manner. Accordingly, one skilled in the art would not readily utilize methods for swelling a diploid nucleus with a haploid nucleus, and vice versa.

Thus, the disclosure of Samocha-Bone cannot readily be applied to the present invention. Particularly, there is no reason to combine the teaching of Samocha-Bone with that of Wangh because one skilled in the art would not have considered the disclosure of Samocha-Bone concerning sperm nuclei to be applicable to a method involving diploid nuclei.

As further evidence of the differences in treating diploid and haploid cells in the art prior to the present invention, Wangh in fact discloses different protocols for treating diploid and haploid nuclei. Example I of Wangh (Nuclear Activation at col. 11-22) details methods for treating diploid nuclei that include a reducing step, followed by salt extraction, then heparin treatment and then treatment with a protease. Example IV of Wangh (Activation of Mammalian Sperm at col. 24-25) details methods involving haploid nuclei including lysolecithin treatment, then trypsin treatment, followed by a reducing agent and then incubation with CSF extract. The differences in the steps of treating different types of cell illustrate that one skilled in the art regards the cell types to require different steps and would not have considered the teachings of a reference dealing with sperm/haploid nuclei to be applicable to a method involving somatic/diploid nuclei.

The Office Action also alleges that Wangh discloses the same treatment of the nucleus to induce swelling as the claimed invention. However, Wangh and Samocha-Bone, disclose that isolated haploid nuclei should be subjected to a reducing reaction. Wangh discloses treating haploid nuclei with, amongst other ingredients, DTT (dithiothreitol), and Samocha-Bone discloses treating haploid nuclei with, amongst other ingredients, 2-mercaptoethanol. Both DTT and 2-mercaptoethanol are reducing agents.

Accordingly, from these two prior art references one skilled in the art would have considered the use of a reducing agent essential for decondensing (haploid) nuclei. Furthermore, while Samocha-Bone discloses the use of heparin, a polyanion, the absence of polyanions from

the methods disclosed by Wangh for treating sperm nuclei would have lead one skilled in the art to interpret its presence as non-essential.

Therefore, the disclosures of Samocha-Bone and Wangh cannot be combined to arrive at the method of claim 13, as one skilled in the art referrring to Wangh and Samocha-Bone and wishing to establish a protocol to decondense isolated nuclei would have considered the use of a reducing agent essential (with or without the additional feature of heparin). The claims of the present application do not include the use of a reducing agent. As discussed above, the claimed invention is a complete and closed series of steps useful for preparing a reconstituted embryo. Accordingly, the claimed invention provides a method for reconstituting an embryo that does not include a reducing agent. As a result, the claimed invention is not obvious over the combined methods disclosed by Wangh and Samocha-Bone.

Moreover, the claimed invention, as amended, recites that the nucleus be treated and consequently swelled prior to transferring followed by activation. In contrast, the methods of Wangh do not disclose this sequence of steps. Wangh discloses that both swelling and activation of the nucleus occur prior to transfer into the recipient oocyte (*see* Wangh at columns 31-32). Accordingly, the claimed method is not anticipated or obvious over the steps disclosed by Wangh.

In view of the differences in the sequence of steps, the lack of egg extract for activation, the absence of a reducing agent, the claimed invention of a closed and complete method for reconstituting an embryo is novel and non-obvious over the cited references. It is therefore respectfully requested that this rejection be withdrawn.

Conclusion

The foregoing amendments and remarks are being made to place the application in condition for allowance. Applicants respectfully request entry of the amendments, reconsideration and the timely allowance of the pending claims. A favorable action is awaited. Should the Examiner find that an interview would be helpful to further prosecution of this application, she is invited to telephone the undersigned at their convenience.

If there are any additional fees due in connection with the filing of this response, please charge the fees to our Deposit Account No. 50-0310. If a fee is required for an extension of time

under 37 C.F.R. §1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,
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DNA Packaging in Sperm

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The chromatin constitution of spermatozoa differs from that of all other cell types. The DNA is mainly associated with basic chromosomal proteins that are different from the otherwise universal histones, allowing packaging of the chromatin in a much smaller volume. Other biological functions in addition to a higher degree of chromatin compaction may be related to the difference in chromatin constitution but their nature is speculative.

Advanced article

Article Contents

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Introduction

In mature male germ cells, the chromatin conformation differs from that of other cell types (Hecht, 1998). The deoxyribonucleic acid (DNA) is packed more tightly than and in a way different from metaphase chromosomes. This is achieved with the aid of male germline-specific chromosomal proteins. Such proteins are characterized by their particularly high content of basic amino acids (arginine, histidine and lysine), which compensates for the highly negative charges of the DNA and therefore allows a higher degree of compaction. The best known of such basic proteins are the protamines, a class of small basic proteins found in many eukaryotes, but with only a limited degree of sequence similarity between different organisms (Lewis *et al.*, 2003). Protamines are not universal in eukaryotic spermatozoa. In some phylogenetic groups, for example, a sperm-specific histone H1 protein can substitute the functions of the normal core histones. This implies that the mode of packaging of DNA in sperm nuclei is not under a stringent control by specific protein conformations as is in other cell types with the aid of the core histones. The requirements for tight packaging of the chromatin in sperm nuclei can be fulfilled by different basic proteins since no specific genomic activities, such as a cell cycle-controlled DNA replication, occur in the metabolically relatively inactive sperm nucleus. The biological reasons for sperm chromatin being organized differently from that of somatic cells are, however, still as unclear as the details of the molecular and structural organization of sperm chromatin. **See also:** Chromosomes: higher order organization; Chromosome mechanics

The special character of sperm chromatin was recognized more than 100 years ago. Friedrich Miescher discovered small basic proteins, the protamines, as major constituents of sperm nuclei in salmon (called 'nuklein'). Sperm nuclei contain chromatin of a much higher level of compaction than nuclei of any other cell type. Electron microscopy also revealed that the fine structure of sperm chromatin differs from that of chromatin in other cells. It is, at least in its major portion, not organized in nucleosomes.

Structural Properties of Sperm Chromatin

The size and shape of sperm nuclei vary widely in different organisms. Although in some species sperm heads are very prominent parts of the spermatozoa, sperm nuclei of most organisms are much smaller than those of the somatic cells of the same organism, and smaller than one would expect from the combined volumes of a haploid metaphase chromosome set. For example, for the fruit fly *Drosophila melanogaster*, the volume of a mature sperm nucleus has been estimated to be one-third of the volume expected from the volume of the metaphase chromosomes and in vertebrates the DNA is packaged in a volume less than 5% of that of a somatic cell (Cho *et al.*, 2001).

Detailed analysis of the chromatin ultrastructure in sperm reveals properties that are different from those of the structure of chromatin in mitotic cells. Generally, the packing density of chromatin is much higher than in any other cell type. In contrast to other cell types, however, one cannot draw generally valid conclusions concerning a specific type of structural organization of sperm chromatin. Depending on the specific organism, there may be thick fibres of a granular or globular sheet-like arrangements, or even an almost crystalline mode of chromatin packing. Only the absence of a nucleosomal organization in sperm chromatin, except possibly for minor portions of the chromatin, appears to be common to all organisms. **See also:** Mitosis; Nucleosomes: structure and function

Special arrangements of the chromosomes were established by traditional methods, in particular autoradiography. In the sperm nucleus of the newt *Plethodon*, the chromosomes are arranged in a specific order. Immunohistology and *in situ* hybridization techniques, together with an increasing number of molecular probes, have more recently provided much more refined tools to study the location of specific chromosomes or sections of chromosomes. An

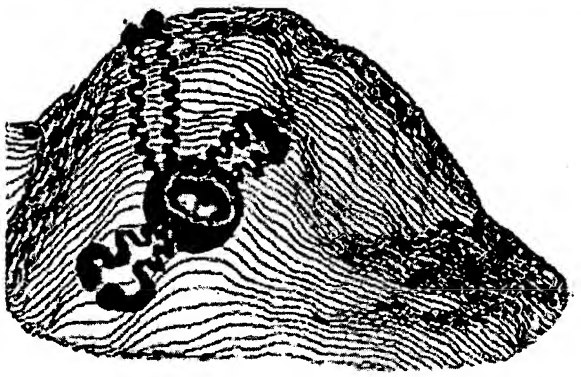

Figure 1 Architecture of a human sperm nucleus. Three of the four telomeres of the two homologues of chromosome 2 are visible. They are attached to the nuclear membrane. The centromeres of chromosome 2 and of other chromosomes form a chromocentre inside the nucleus. From Zalensky *et al.* (1995).

ordered arrangement of chromosomes in sperm heads has been shown for mammals (Watson *et al.*, 1996), and even the localization of small chromosome sections can be shown. In human sperm nuclei (**Figure 1**), the centromeres are fused into a centrally located chromocentre, while the telomeres are assembled within a limited region of the posterior periphery of the nucleus (Zalensky *et al.*, 1995). The telomeres associate to dimers and in some species stay in contact with the nuclear membrane. This type of structure seems to be generally valid for mammalian spermatozoa, but in some groups of mammals telomeres form tetramers or higher-order associations. **See also**: Centromeres; DNA synthesis: autoradiography and BrdU staining; Fluorescence *in situ* hybridization; Telomeres

Comparable studies on the details of nuclear substructure in relation to individual chromosomes have so far only been carried out in a small number of species; it is at present impossible to say whether they represent a comprehensive picture of the structural organization of sperm chromatin or whether they just reflect some of the possible variants.

Molecular Components of Sperm Chromatin

For a long time it has been known that the protein components of chromatin in sperm nuclei differ from those of all other cell types. It seems safe to conclude that the chromatin of mature sperm never has the same basic structural organization as displayed in somatic cells or in cells of earlier stages in the male germline, i.e. a core histone-controlled nucleosomal configuration. The DNA-associated proteins in sperm chromatin are more uniform in their composition, and they are characterized by a high content of basic amino acids. The best known – and probably most widely distributed – classes of such proteins are the protamines (Lewis *et al.*, 2003). Protamines are small basic proteins with a characteristically high content of arginine. The arginines are often found in clusters. In contrast to the core histones (H2A, H2B, H3 and H4), protamines are not evolutionarily well conserved and display a widely divergent amino acid sequence in different organisms, with only minor conserved sequence sections. In humans, mice and some mammals, two different protamines (P1 and P2) are found. The genes of these two protamines are present in most if not all mammals, but they are expressed only in some species. If expressed, their relative proportions can vary in a wide range but are tightly controlled within a genus. The otherwise universal protein components of chromatin, the core histones, are not present, or are present only in small amounts, if protamines are constituents of the chromatin. Histones in human sperm nuclei represent 20% or less of the total basic nuclear proteins and protamines of at least 70%. In teleost fish, histones are fully replaced by protamines, and in some molluscs 92% of the basic sperm nuclear proteins are represented by one protamine type, the remaining few per cent by a sperm-specific histone H2B variant (see Wouters-Tyrou *et al.*, 1998). **See also**: DNA topology: fundamentals; Supercoiled DNA: structure

However, protamines can by no means be considered as exclusive or even universal sperm proteins. In some organisms, the packaging function for DNA is assigned to sperm-specific histone variants. For example, in some molluscs, a histone variant similar to histone H1 takes over the function of protamines in sperm chromatin. Similarly, sperm-specific histone H1 variants are found in the sea anemone (*Urticina crassicornis*, Coelenterata). These histone H1 variants are similar to the somatic histone H1 in their amino acid sequence and structure, displaying a compact globular configuration. They are also similar to the avian histone H5, which is found in the compacted, inactive chromatin of avian erythrocytes. In some teleostean fish, two histone-like proteins are found together with one protamine in the mature sperm.

An evaluation of the, fairly limited, information available on the chromosomal protein content of sperm nuclei of different organisms leads to the view that no uniform and generally valid mechanisms exist to achieve a high degree of packaging in the mature sperm. The general requirement to pack sperm chromatin more tightly than chromatin of other cell types can be achieved in different ways: either by the use of sperm-specific small basic proteins like protamines or by sperm-specific variants of histones, which during evolution have probably been created by gene duplications. In fact, the different types of sperm chromatin proteins may have an evolutionary relationship (Lewis *et al.*, 2003). The universal somatic core histones always appear to be substituted by species-specific variants. This replacement is not surprising as cell-cycle-regulated histones are no more produced after the last S phase during meiosis and cannot therefore readily contribute to

the (meiotic and postmeiotic) compaction of the chromatin (Hennig, 2003).

Replacement of Histones in the Germline

Sperm-specific nuclear proteins like protamines or histone variants appear usually late in the postmeiotic development of male germ cells when the nuclei are metabolically inactive. The fundamental question is why in male germ cells such a substitution of the somatic histones is necessary.

It has often been argued that the chromatin in sperm cells must be protected against environmental influences leading to mutations. This could hold true especially for organisms with external fertilization. A tight packing of the chromatin may protect it more than a more loose conformation. Whether this is really the case, however, has never been experimentally proven. In fact, the few available data argue against this assumption. For example, in *D. melanogaster*, mutation frequencies are higher in spermatozoa than in some earlier stages of the male germline that have less compact chromatin. In addition, one must expect that the level of mutagenic effects on compacted chromatin varies for different types of mutagens. An opposite indication has, however, been obtained for medeca (*Oryzias latipes*). In this species, protamines may be required to protect the sperm chromatin against distortion by low osmotic pressure after the release of sperm into the water (Shimizu *et al.*, 2000). **See also**: Mutagenesis mechanisms

It seems appropriate to search for other functional reasons that might require an exchange of the histones with other proteins responsible for a less complex nucleoprotein structure. One such reason might be the necessity for the compacted chromatin of the male pronucleus in the zygote to decondense very rapidly and uniformly and to take up histones to re-establish the nucleosomal chromatin conformation. It has been suggested that the telomeric sections of the chromosomes, which persist through the postmeiotic development of the sperm nucleus in a nucleosomal structure, are important initiation points for decondensation (Zalenskaya *et al.*, 2000). A nucleosomal conformation of DNA is essential for the initiation of transcription in the early development .The required structural change of the chromatin of the male pronucleus can probably be most easily achieved if the DNA is assembled in a uniform chromatin structure without much longitudinal structural differentiation into different chromosomal domains.

Another reason to abandon the nucleosomal structure of the chromatin may relate to the functional importance of differential packaging of chromatin in different developmental situations. In the male germline, the cell has first to follow, and complete, a differentiation programme that leads to the development of one of the most complex cells in eukaryotes, the spermatozoon. This differentiation process is usually not at all, or not to a large extent, supported by subsidiary cells. It is therefore mostly determined by the specific differentiation programme of the germ cell. After completion of this programme, however, the genome must be reset to a more or less undetermined regulatory state to meet the requirements of the many different differentiation programmes set up in the developing embryo. Such a change in the differentiation programme of a cell, which one might designate as a 'dedifferentiation' of the genome (i.e. removal of differentiation signals), does not usually take place in other differentiated cells of multicellular organisms. Chromatin that has received determining signals for a distinct differentiation pathway cannot be reprogrammed easily. In the male germline, however, this dedifferentiation of the genome is essential as the embryo cannot make use of a paternal genome programmed to develop a spermatozoon. How this dedifferentiation occurs is still unknown. It is an attractive idea that the substitution of the histones by other proteins, and the creation of a more uniform chromatin structure, is the key for understanding the mechanisms involved in the reprogramming of the genome. The mechanisms required to establish differential demethylation of maternal and paternal DNA in the early embryo of mammals may also be based on a special constitution of the chromatin of the paternal pronucleus (Barton *et al.*, 2001).

Histones are now well established as regulatory elements involved in differential gene regulation. This is, in part, achieved by differential modification of the histone proteins, for example by phosphorylation, methylation or acetylation. In differentiating cells, histones display complex and specific patterns of such modifications, which change in a strictly defined sequence. In this way, they participate in the determination of transcriptionally active and inactive domains of the chromatin. Interactions with other chromosomal proteins play an important role in this context. The type and amount of modifications, in particular in the *N*-terminal sections of the histones (see the review by Lachner *et al.*, 2003) control this interaction with other proteins. The terminal sections of the histones penetrate to the outside of the nucleosome particles and are therefore available for modification and for interactions with other chromosomal proteins. A complete substitution of all histones could result in the removal of all such signals and lead to a deregulated and inactive state of the chromatin. A uniform constitution of the chromatin in postmeiotic stages may moreover facilitate or enhance the capacity of DNA mechanisms removing breaks in the DNA (Kierszenbaum, 2001). This becomes particularly important as the normal cell-cycle control mechanisms, which include an efficient check for DNA integrity, are absent in the postmeiotic stages. It has recently become evident that DNA breaks and their repair occur with high frequency during the postmeiotic development and may have important consequences in the context of genetic diseases (McMurray and Kortun, 2003). **See also**: Chromatin remodelling and histone modification in transcription regulation

Histone Variants and Protamines

That histone variants can enhance the compaction of chromatin beyond that induced by the formation of nucleosomes is documented by the occurrence of specific transition proteins with features of histones. One might then ask why in some organisms histone variants – in particular, histone H1 variants in the sea anemone, histone H2B variants in sea urchins or histone H1 variants in molluscs – can fulfil the same purpose as protamines in other organisms. The answer is most likely that these variant histones cannot reconstitute a normal nucleosome structure, at least as long as the other core histones are absent. Their structural differences to the core histones may qualify them to take over similar functions as protamines. In fact, chromosomal proteins have been identified which may represent evolutionary transition forms between histones and protamines (Lewis *et al.*, 2003). A remarkable example is the sperm-specific histone H1 in *Mytilus* that has developed protamine-like structural properties. Histone H1 is not a constitutive part of the nucleosome but fulfils – in a manner still not clearly understood – functions in chromatin condensation. Histone H2B is more variable than the other core histones, especially more than the histones H4 and H3, and it therefore appears to be more flexible in its specific contributions to the structural organization and maintenance of chromatin. This may allow an evolution towards functions other than that of a nucleosomal core histone. A more general consideration is that the nonstringent requirements of DNA packaging in the sperm head might qualify a variety of proteins with some common basic features to be used for the postmeiotic packaging of the genome (Hennig, 2003). The evolution of a variety of protamines and the pattern how they are used in different phylogenetic groups seems to support such a view.

Occurrence of Transient Intermediates

It is a feature of particular interest that the substitution of the, otherwise universal, structural organization of the DNA in nucleosome chains with the aid of the core histones by sperm-specific proteins, at least sometimes, is achieved in several steps. In mammals, transient 'substitution' or 'transition' proteins participate stepwise in this process. Such substituting proteins are specific for the male germline. Transition proteins have a chemical status intermediate between histones and protamines. They are in general more basic than histones but less basic than protamines. Usually, they become expressed in a strict sequential order during the late first meiotic prophase and after meiosis, i.e. at the time of the beginning of compaction of the chromatin in the spermatids (see Zhao *et al.*, 2004). We are presently far from understanding the detailed mechanisms involved. An understanding of the details of this process may provide a key to understanding the general processes responsible for creating and maintaining a particular chromatin conformation.

An important question is why the chromatin substitution in some organisms, such as mammals, occurs in a series of several steps, rather than by a simple replacement of the somatic core histones in one step as it appears to be the case in other organisms. We have no answer to this question, but can only speculate on the reasons. Arguments may be derived from the observation that most vertebrates appear to have genes for several protamines, but that often only some or even one of them are used. Nevertheless, if a particular expression pattern for protamines is established, a compensation of the absence of one of the protamines by another seems not admissible. Also the substitution of protamines by those derived from other species seems not to be possible (Maleszewski *et al.*, 1998). The most immediate interpretation of these observations is that the specific sequence and composition of substituting proteins has developed in a concerted way and cannot easily be changed by substitution or deletion of single components of the system (Cho *et al.*, 2001). Thus, although there is no general tight restriction of the kinds of chromosomal proteins packaging sperm chromatin, one might assume that an established system is much less flexible than one would expect. This might imply that the packing of chromatin in sperm has to account for a number of different underlying tasks.

In mammals, a limited amount of transcriptional activity of the genome is observed in early spermatid nuclei. Most of this transcription is related to the production of protamines, which must apparently be kept away from the chromatin in earlier stages. Transition proteins, which appear to be less efficient than protamines in compacting chromatin, may induce a more gradual compaction than protamines. This could be important from the view of a gradual shutdown of the transcription in the male germline. The transcription of some genes in the haploid postmeiotic genome is under the regulatory control of other proteins, which might include specific high-mobility group (HMG)-box containing proteins. **See also**: Reproduction in mammals: general overview

Transition proteins TP1 and TP2 of the mouse appear to some extent to overlap in their functions, as deletion of either of the proteins in mice does not lead to sterility, although lower fertility and deviating phenotypes of sperm are observed (Zhao *et al.*, 2004). This does not exclude specific tasks for each of these proteins. An example is TP1, which is probably involved in the repair of DNA breaks (Kierszenbaum, 2001). Moreover, additional proteins are involved in the deposition of the transition proteins and protamines. In the absence of calmodulin-dependent protein kinase IV (Camk4), the replacement of TP2 is delayed and protamine 2 is deposited in a reduced amount. This implies more complex events in the chromatin during the substitution process. The observation that the injection of round spermatids (which have no protamines) of mice can

induce the embryonic development suggests that the final packaging of the genome is functionally not essential for its performance in the zygote (Kimura and Yanagimachi, 1995). The importance of the chromatin rearrangements during sperm development must hence most likely concern functional aspects of the chromatin rearrangements in the preparation of the genome for fertilization.

Protamines (or other basic proteins) determine the final structure of the chromatin in the sperm nucleus: they bind to the major groove of the DNA (Prieto *et al.*, 1997) and allow the formation of parallel structures by the association of the neutralized DNA strands. The formation of such structures can be supported by the ability to form disulfate bridges between the cysteine residues of the protamines (Bedford and Calvin, 1974). Protamines, however, may also participate in a different way in the organization of the chromatin. Protamine P2 of humans and mice has a zinc-finger motif, which has similarly been found in the sperm-specific histone H1 of sea urchins. It is suggested that such proteins can, comparable to leucine-zipper-containing proteins, interact with DNA by forming a scissor-like structure, which encloses the DNA strand(s). In this way they may tighten the parallel association of DNA strands.

An important aspect of mammalian sperm chromatin is the persistence of up to 20% of histones in the mature sperm nucleus. At least some of these histones maintain a nucleosomal structure of parts of the chromatin (see Zalenskaya *et al.*, 2000). The nucleosomal conformation may be important for the phenomenon of paternal imprinting. Some genes are differentially expressed in the early embryo, depending on the respective parental origin of the individual alleles. This is achieved by an epigenetic control imposed on alleles of a particular (either paternal or maternal) origin. The epigenetic signals are most probably imposed during the primary spermatocyte stage and persist throughout the postmeiotic differentiation processes. It is reasonable to assume that such imprinted genes maintain a specific chromatin structure in the sperm chromatin, which might be characterized by nucleosomes. Gardiner-Garden *et al.* (1998) have obtained experimental evidence for a relationship between chromatin structure and transcriptional activity in early development. These authors have shown that the globin genes activated first during early sperm development, i.e. ε- and γ-globin, are associated with histones, while the globin genes active later in development (β- and δ-globin) are packed more tightly and are associated with protamines. These observations indicate a rather distinct control of the distribution of histones and protamines. They also clearly show that the presence of histones in chromatin is related to the control of differential gene activity. The complete removal of histones would hence possibly also remove epigenetic control signals required for differential gene regulation, and in this way reset the genome for the development of a new organism. One of the functions of transition proteins may be found in the need for a controlled gradual replacement of the histones (see Zhao *et al.*, 2004 for the time course of the expression of transition proteins). The differential association with transition proteins could be used to signal which parts of the chromatin must be covered by protamines when the compaction of the germline chromatin is completed, but in earlier phases of postmeiotic development it may still allow parts of the genome to be transcribed.

See also: Globin synthesis; Imprinting (mammals); Morphological evolution: epigenetic mechanism

Transition Proteins and the Structure of the Sperm Nucleus

The differential association with different substitution proteins of sperm chromatin domains may also be related to the structural organization of the chromatin inside the sperm nucleus. This is implied by the observation that nucleosome-like structures in the chromatin are located close to the nuclear periphery. The telomeres in human sperm nuclei are even better examples, emphasizing a distinct nuclear organization. Telomeres in mammals associate in pairs or in tetramers in a distinct region of the spermatid nucleus close to its periphery, probably attached to the nuclear membrane (Zalensky *et al.*, 1995; Haaf and Ward, 1995). The nucleosomal conformation (Zalenskaya *et al.*, 2000) might be important in this regard as it may support the association with the lamin layer on the nuclear membrane. The centromeres of the chromosomes in human sperm, in contrast, are fused inside the central part of the nucleus, forming a large chromocentre.

Transition proteins may, in addition to their interactions with other proteins, have the task of directly altering the conformation of the DNA: as a consequence, the chromatin structure would be modified. The transition protein 4 (TP4), found in late boar spermatid chromatin, is assumed to support a transition from a negatively supercoiled state of the DNA (in its nucleosomal conformation) by topoisomerase I into a nucleoprotamine conformation, which does not include supercoiled DNA (Akama *et al.*, 1999). Changes in the DNA conformation in connection with chromatin condensation are expected from the earlier observation that inhibition of topoisomerase II prevents chromosome condensation in mitotic nuclei. Besides the availability of different chromosomal proteins that determine the specific condensation in sperm nuclei, their mode of modification is also important. Major changes are observed in the degree of phosphorylation, while methylation and acetylation appear less relevant. Generally, specific phosphorylation has been found in earlier spermatids. The *N*-terminal Ser-Pro-X motifs and Lys-Arg motifs in the sperm-specific histones H1 and H2B in sea urchins are phosphorylated early in spermatid development but become dephosphorylated later, and in late spermatids and

mature sperm, phosphorylation is absent. The *N*-terminal phosphorylation controls the interaction with other chromosomal proteins and hence the chromatin structure. Similarly, in other organisms, a dephosphorylated state of histone H1 has been found in highly condensed chromatin, for example, the chromatin in avian erythrocytes contains dephosphorylated histone H1.

Other modifications of the histones are also removed during the final stages of chromatin compaction. The transition protein TH2B in rat meiotic cells and spermatids is hypermethylated and there are no reports of acetylated sperm proteins. **See also:** DNA topology: supercoiling and linking

Conclusions

Our knowledge of the structural and functional organization of the compacted chromatin in male germ cells is expanding but still very limited. It is not known why such a high level of compaction is required, nor is the detailed mechanism understood of how, and with which structural properties, such a compaction is achieved. Several requirements seem to be interrelated and may all have contributed to the development of the unique condensation behaviour of chromatin in male germ cells. They include deregulation of the genome before the onset of the developmental processes in the new organism after fertilization, the introduction of epigenetic information required for paternal effects in the embryo, the need for DNA repair as has recently emerged, as well as structural demands of the sperm head related to fertilization and to the subsequent rapid decondensation and repackaging into a nucleosomal conformation of the genome after fertilization.

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